Supporting Information

Anti-staphylococcal Activity of Injectable Nano Tigecycline/Chitosan-PRP Composite Hydrogel Using *Drosophila melanogaster* Model for Infectious Wounds

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1. Kinetic Modeling of *In Vitro* Tigecycline Release. The kinetics of tigecycline release from nanoparticles and gel systems were evaluated by different theoretic models. The kinetic

models like zero order, first order, Higuchi and KorsmeyerPeppas were used for kinetic study. The four kinetic models were studied by plotting specific ordinate and abscissa.[1]The linearity (R^2) and slope (n) of all the models were determined to find the tigecycline release mechanism (**Table S1**).

Model dependent method	**Abscissa (X axis)**	**Ordinate (Y axis)**
Zero order	Time	Cumulative amount of drug released.
First order	Time	Log_{10} (cumulative percentage of drug remaining).
Higuchi	Square root of time	Cumulative percentage drug release.
Korsmeyer-Peppas	Log_{10} (Time)	Log_{10} (Cumulative percentage of drug release).

Table S1: Abscissa and ordinate for different drug release models

Model	tg-ChNPs		tg-ChPRP Gel		tg-ChNPs-ChPRP Gel	
	R^2	n	R^2	n	R^2	n
Zero order	0.673	1.551	0.720	0.658	0.952	0.403
First order	**0.767**	-0.053	**0.934**	-0.212	**0.996**	-0.048
Higuchi	0.673	7.755	0.720	13.152	0.952	8.078
Korsmeyer-Peppas	**0.810**	**0.033**	**0.761**	**0.041**	**0.976**	**0.118**

Table S2: The tigecycline release kinetics of tg-ChNPs, tg-ChPRP gel and tg-ChNPs-ChPRP gel.

2. Whole Blood Clotting Study. The hemostatic potential of the prepared Ch gel, ChPRP gel (5 mg/g PRP) and tg-ChNPs-ChPRP(10 µg/mg tg-ChNPs and 5 mg/g PRP) gels were studied using human blood. Briefly, 1 g of gel systems were added to 50 mL falcon tubeand

pre-incubated in 37ºC for 1 h. After the incubation, 200 µL of citrated whole blood was added on the surface of the gels followed by the addition of 20 µL of 0.2 M calcium chloride ($CaCl_2$). The blood without any gel and $CaCl_2$ was used as control. The tubes were incubated at 37°C for 10 mins. 25 mL of double distilled water was added drop wise to the tube without disturbing the clot formed. Subsequently, 10 mL of the solution from each tube was collected carefully and centrifuged at 1000 rpm for 2 min (HettichEBA21). The supernatant was collected and the presence of hemoglobin was analyzed by measuring the optical density at 540 nm using a microtiter plate reader (BioTekPowerWave XS). The whole blood-clotting index (BCI) was calculated using the following equation.

$$\text{BCI (\%)} = \frac{\text{O.D}_{\text{TEST}}}{\text{O.D}_{\text{CONTROL}}} \times 100$$

3. Hemolysis Assay. The hemolysis assay was performed to measure the extent of hemolysis caused by the nanoparticles or gel when it comes into contact with human blood.[2] Different quantities of hydrogel (50 mg and 250 mg) were added to 1.5 ml vial containing 500µL of diluted blood (1:9 in PBS). The tubes were further incubated 37ºC incubator for 24 hrs. 0.1% (v/v) Triton -X 100 treated blood samples were served as the positive control and PBS treated blood samples were served as the negative control. After the incubation tubes were centrifuged at 3000 rpm for 10 mins (Minispineppendorf centrifuge) and the absorbance of supernatant were measures at 540 nm using microtiter plate reader.[3,4]The percent of hemolysis was calculated using following equation:

$$\text{Hemolysis (\%)} = \frac{\text{O.D}_{\text{TEST}} - \text{O.D}_{\text{NEGATIVE CONTROL}}}{\text{O.D}_{\text{POSITIVE CONTROL}} - \text{O.D}_{\text{NEGATIVE CONTROL}}} \times 100$$

4. Cell Culture. Mouse fibroblast (L929) cell lines were maintained in Dulbecco's Modified Eagle Medium (DMEM) media supplemented with 10 % fatal bovine serum at 37°C under 5% CO_2 in a standard animal cell culture incubator.[4,2]

4.1. Cell Viability Studies. The cell viability of 50 mg and 250 mg of tg-ChNPs-ChPRPgels (10 µg/mg tg-ChNPs and 5 mg/g PRP) were tested using Alamar blue assay.[4]Briefly, 10,000 L929 cells in 1 ml of complete DMEM medium were added in to the wells of 24 well plate containing test samples. The plates were incubated in 37ºC CO_2 incubator for 24 and 48 h. The untreated and 0.1% (v/v) triton X-100 treated cells were used as positive and negative control respectively. Following the incubation the culture medium was replaced with 10% (v/v) alamar blue in DMEM and further incubated for 4 h.[3] The absorbance of the solution from each well was measured at 570 nm and 600 nm using microtiter plate reader and the per cent cell viability was measured using following equation.

$$\text{Cell viability (\%)} = \frac{\text{O.D}_{\text{TEST}} \text{ at 570 nm - O.D}_{\text{TEST}} \text{ at 600 nm}}{\text{O.D}_{\text{POSITIVE CONTROL}} \text{ at 570 nm - O.D}_{\text{POSITIVE CONTROL}} \text{ at 600 nm}} \times 100$$

4.2. Cell Proliferation. 500 mg of tg-ChNPs-ChPRPgel (10 µg/mg tg-ChNPs) with different concentrations of PRP (0, 0.5, 1.5 and 5 mg/g) were placed on the bottom of 24-well plates. 1 mL of L929 cell suspension in complete cell culture medium ($1X10^3$ cells/mL) were added to the wells and incubated for 24, 48 and 72 h to evaluate their proliferation. Once the speculated culture period was reached, the culture medium was replaced by 10% alamar blue in basal medium and incubated for 4 h at culture conditions. The absorbance of the medium was measured at 570 and 600 nm using microtiter plate reader. The cell number was calculated from concurrently plotted standard curve with L929 cells.

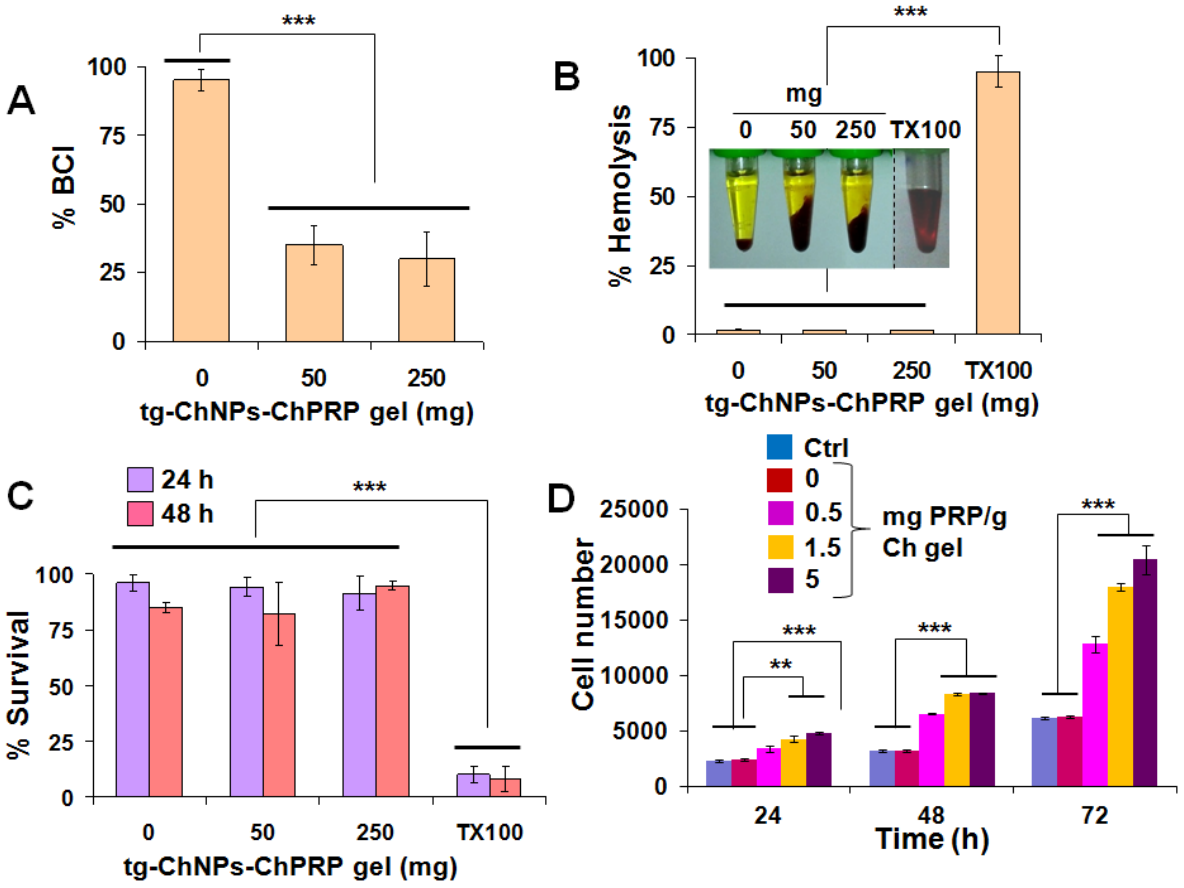

Figure S3: (A) Blood-clotting ability of tg-ChNPs-ChPRP gel. (B) Photograph and bar diagram representing the hemocompatibility of human RBCs treated with tg-ChNPs-ChPRPgel. The red color of the solution is due to the release of hemoglobin from the damaged erythrocytes, and the red pellets at the bottom of the eppendorf tubes are intact erythrocytes precipitated by centrifugation. Triton X 100 treated samples were served as the positive controls. (C) Cell viability assay of the hydrogel against L929 cell line. (D)Proliferation of L929 cell in the presence of tg-ChNPs-ChPRP gel with different concentrations of PRP.

5.1. Antibacterial Activity.

5.1.1. Bacterial Strains and Culture Conditions. Green fluorescent protein (SApC*gfp*)[5] expressing *S. aureus* strain SA113 (ATCC 35556) was grown on LB agar plate and in LB broth at 37 ºC.[4]Bacterial culture medium was supplemented with 20 µg/mL chloramphenicol.

In liquid culture *S. aureus* bacterial cells were grown at 120 rpm shaking (Multitron Standard, Infors HT).

5.1.2. *In Vitro* Antibacterial Activity.

5.1.2.1. Agar Well Diffusion. The antibacterial activity of 100 mg of Ch gel, ChPRP (5 mg/g PRP), tg-Ch, tg-ChPRP(5 mg/g PRP), tg-ChNPs-Chand tg-ChNPs-ChPRP gel (5 mg/g PRP) with different concentrations of tigecycline (0.01, 0.05, 0.1 and 0.5 µg/mg) were visualized by the agar well diffusion assay in the form of bacterial inhibition zone.[6] 50 µL of McFarland standard-0.5 was spread on the LB agar plate using a sterile L rod. Later 8 mm diameter wells were made using biopsy punch. The test samples were added into the wells and incubated in 37ºC for 24 h. The inhibition of bacterial growth around the wells was measured.

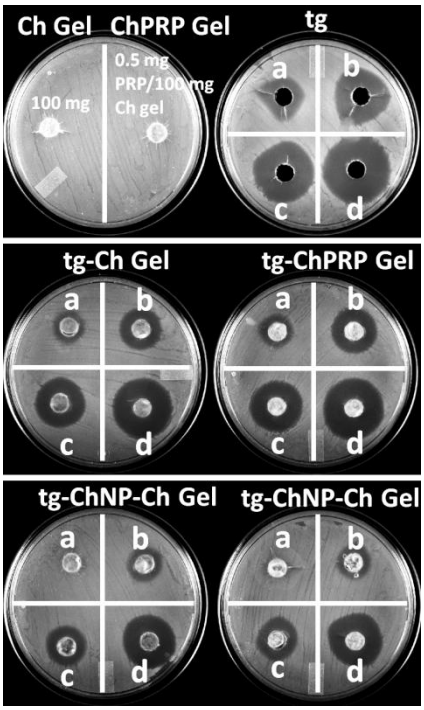

Fig.S4: Agar plate diffusion assay demonstrating anti-microbial activity of Ch gel, ChPRP gel, tg,, tg-Ch gel, tg-ChPRP gel, tg-ChNPs-Ch gel and tg-ChNPs-ChPRP gel at different concentrations of tigecycline. Amount of tg in the wells: (a) - 1 µg, (b) - 5 µg, (c) - 10 µg and (d) 50 µg

5.1.2.2. Broth Dilution Assay. The Minimum Inhibitory Concentration (MIC) of gels was tested using broth dilution assay. [3,4]The tg-ChPRP and tg-ChNPs-ChPRP gels with different concentrations of tigecycline (0, 0.002, 0.02, 0.1, 0.2, 0.4 and 0.6 µg/mg) and 5 mg/g of PRP were added to 5 mL of LB broth containing 5 X 10^6 CFU of *S. aureus* and incubated for 12 h in 37 ºC shaking incubator. After the incubation period the broth was serially diluted and spread plated on LB agar plates. The plates were incubated at 37°C overnight and inspected for colony formation. The number of colonies formed on the LB agar surface were counted and compared with the control.

5.1.3. *Ex-Vivo* Antibacterial Activity. The antibacterial activity of the gels against *S. aureus* on the porcine skin surface was studied. [3,4]The skin excised from porcine ear was washed thrice in PBS and cut in to 1 cm^2 (1 cm X 1 cm) pieces. The ear skin was surface sterilized by incubating in 70% (v/v) isopropanol for 5 min. The air dried ear skin after alcohol treatment was transferred into petri dishes. 10^6 CFU of *S. aureus* in 20 µL was added on the skin surface and incubated for 1 h in 37°C. Next, 1 g of ChPRP (5 mg/g PRP), tg-ChPRP (1 µg/mg tg and 5 mg/g PRP) and tg-ChNPs-ChPRP (10 µg/mg tg-ChNPs and 5 mg/g PRP) gels were added on the infected skin surface and incubated further for 5 h. After incubation, the skin was transferred into separate tubes containing 3 mL of sterile PBS and the tubes were vortexed vigorously for 5 min to detach the bacteria from skin surface. The PBS solution in the tubes were further serially diluted and plated on LB agar plates and incubated for 24 h. The number colonies formed on incubation was counted and compared with the negative control.

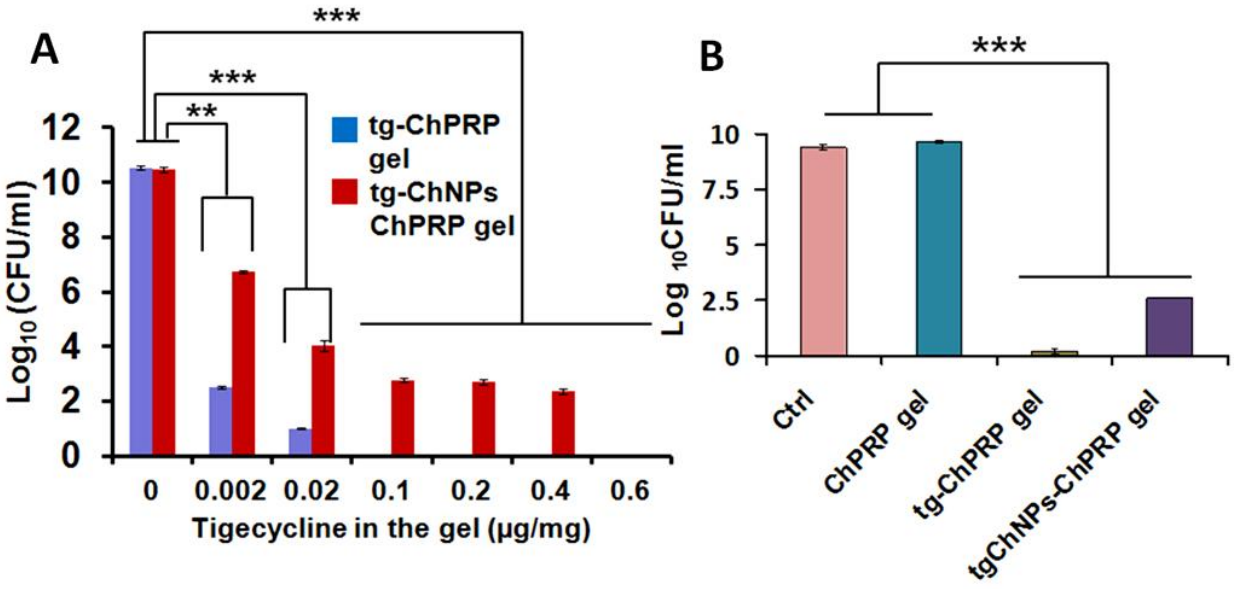

Fig.S5: (A) *In-vitro* antibacterial activity of tg-ChPRP and tg-ChNPs-ChPRP gel with different concentrations of tg and tg-ChNPs.(B) *Ex vivo* antimicrobial activity of ChPRP gel, tg-ChPRP gel and tg-ChNPs-ChPRP gel on *S. aureus* infected porcine ear skin.

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